SOP For Long Oligonucleotide Arrays: Target Preparation & Hybridization Using Total RNA (August 19, 2005)

I. cDNA Generation (SuperScript™ Indirect cDNA Labeling Kit)

Prepare separate cDNA labeling reaction for each fluorescent dye you wish to use.
A "master mix" (step 4) can be made, and the reaction increased up to 5X if needed.

1. Make dilution of 10 µg of total RNA in 16.0µl of DEPC water.
2. Add 2.0µl of 2.5µg/µl anchored oligo $d(T)_{20}$ primer.
3. Incubate at 70°C for 5 minutes. Cool on ice for at least 1 min.
4. Combine the following components for each sample in a sterile, RNase/DNase-free microcentrifuge tube:
    a. 6.0µl of 5X First-Strand buffer
    b. 1.5µl of 0.1 M DTT
    c. 1.5µl of 10mM dNTP mix
    d. 1.0µl of RNaseOUT™ (40 U/µl)
5. Add the mixture to the annealed primer and RNA.
6. Add 2µl of 400 U/µl SuperScript™ III RT and incubate at 48°C for 2hrs.
7. Incubate at 70°C for 5 minutes to stop reaction.
8. Cool down by spinning in a microcentrifuge at maximum speed for 1 minute.
9. Add 2µl of 2 U/µl RNase H and incubate at 37°C for 20 min.
10. Add 0.5 µl of 0.5M, pH 8.0 EDTA, mix well and proceed with purification.

II. cDNA purification: (QIAGEN MINElute Purification Kit)

1. Add 100 µl of Binding buffer ***PB*** to RT reactions and mix well.
   Note: Can add a maximum of 2 reactions per column.
2. Apply to separate spin columns. Incubate for 1 minute.
3. Spin for 1 min at full speed.
4. Discard flow-through.
5. Add 500 µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
6. Spin for 1 min at full speed.
7. Discard flow-through.
8. Repeat wash step.
9. Discard flow-through.
10. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
11. Place columns in a fresh 1.5ml microcentrifuge tubes.
12. Add 10 µl of Elution buffer directly to the membrane. (20 µl of Elution buffer if 2 rxns were put on the column).
13. Incubate for 1 min.
14. Spin for 1 min at full speed.
15. Apply flow-through back on membrane.
16. Incubate for 1 min.
17. Spin for 1 min at full speed.
18. Discard columns, spec on Nanodrop to determine cDNA concentration.
19. Dry down in SpeedVac for 15 min at medium temp. DO NOT OVERDRY!

III. NHS-ester Containing Dyes Coupling Reaction (Pharmacia)*:*

1. Resuspend cDNA pellet in 5µl of 2x coupling buffer. (If pellet was over dried gently heat at 37° C for 15 minutes to aid in the resuspension process.)
2. The first time a tube of dye is used, resuspend in 45µl DMSO. Use DMSO provided with the kit.
3. Add 5µl of the resuspended monofunctional reactive dye to cDNA.
4. Mix thoroughly by gently pipetting up and down.
5. Incubate for 1 hour at room temp in the dark, flicking the tubes occasionally.

*IV. **Dye-Coupled cDNA Purification:*** (QIAGEN MINElute Purification Kit)

1. Add 10µl of 3M Sodium Acetate, pH 5.2 to each RT reaction, mix well.
2. Add 100 µl of Binding buffer ***PB*** to RT reactions and mix well.
3. Apply each RT reaction to separate spin columns.
4. Incubate for 1 min.
5. Spin for 1 min at full speed.
6. Discard flow-through.
7. Add 500 µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
8. Spin for 1 min at full speed.
9. Discard flow-through.
10. Repeat wash step.
11. Discard flow-through.
12. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
13. Place columns in a fresh 1.5ml microcentrifuge tubes.
14. Add 20 µl of Elution buffer for 48-pin print.
15. Incubate for 1 min.
16. Spin for 1 min at full speed.
17. Apply flow-through back on membrane.
18. Incubate for 1 min.
19. Spin for 1 min at full speed.
20. Can spec using Nanodrop to determine labeling efficiency and cDNA concentrations.

*V. **Pre-hybridization:*** *(should start approximately 2 hours before setting up hybridization)*

Pre-hybridization buffer = 5X SSC, 0.1% SDS and 1% BSA. (Can make 10% BSA stock and filter before use or purchase pre-filtered BSA; store pre-hyb buffer at -20° C and thaw only once, warm to 42° C prior to use.)

1. Apply 80 µl of pre-hybridization buffer to the array and incubate for 42° C for 1 hour.
2. Wash off the pre-hybridization solution by rapidly plunging the slide in distilled water for 2 mins, then transfer slide to 100% isopropanol for 2 mins.
3. Spin slide 3 minutes / 650 rpm to to air dry completely prior to use. (NOTE: Do not exceed 1 hour after pre-hybridization/drying before setting up hybridization.

*VI. **Hybridization & Wash Procedures***

1. Combine Cy3 and Cy5 labeled targets together (~9 µl recovered for each).
2. Add 1µl COT-1 DNA (8-10 µg/µl) and 1µl poly A (8-10 µg/µl).
3. Denature target at 100^{o}C for 1 minute, then snap cool on ice. Volume should be about 20µl. Bring to a final vol of 40 ul with water
4. Make fresh 2X Formamide hybridization buffer (50% formamide, 10x SSC, 0.2% SDS) and warm to 42^{o}C just before adding to samples.
5. Add 20µl of 3X SSC to wells in hyb chamber to maintain humidity.
6. Add 40 µl of 2X F-hyb buffer to 40 ul samples for 48-pin print.
7. Load 80 µl onto 48-pin print.
8. Incubate overnight 16 hours at 42° C in water bath or hybridization oven.
9. Quickly and carefully remove cover slip and:
    - Wash 2 min at room temp in 2XSSC, 0.1% SDS with occasional plunging.
    - Wash 2 min at room temp in 1XSSC with occasional plunging.
    - Wash 2 min at room temp in 0.2XSSC with occasional plunging.
    - Spin 3 minutes at 650 rpm to dry.
10. Scan ASAP

Recommended Supplies/Suppliers for Microarray Probes and Hybridization

(*August 2005*)

SUPPLIES	SUPPLIER	Catalog No.
SuperScript™ Indirect cDNA Labeling Kit	Invitrogen	L1014-02
Dyes: Cy3 monofunctional reactive dye Cy5 monofunctional reactive dye	 Pharmacia Pharmacia	 PA23001 PA25001
Ribonuclease H	Invitrogen	18021-071
Poly (dA) 40-60	Pharmacia	27-7988-01
COT-1 DNA: Human COT-1 DNA (for human arrays) Mouse COT-1 DNA (for mouse arrays)	 Invitrogen Invitrogen	 15279011 18440016
Coverslips: 48-pin print: Lifterslips™ (25 X 60 mm) mSeries™ (25 X 60 mm)	 Erie Scientific Erie Scientific	 25X60I-2-4789 25X60I-M-5439
Staining Dish/rack (10 slide)	Fisher	08-812
Slide Box (100 slide)	Thomas Scientific	6708-G28
Slide Box (25 slide)	Thomas Scientific	6708-G08
Hybridization chambers: Dual Hyb Chamber Single Hyb Chamber Single Hyb Chamber Deeper hyb chamber to accommodate thicker mSeries™ cover slip: Single Hyb Chamber	 Genomic Solutions Telechem Int., Inc. Corning Telechem Int., Inc.	 JHYB200004 AHC 2551 AHCXD
Hyb Oven	Fisher Scientific	13-247-10
Forceps	Fisher Scientific	10-295
Mini-Elute PCR Purification Kit	Qiagen	28004
High Quality Pre-filtered BSA	Invitrogen	15561-020
Centrifuge with microplate carrier assembly		